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Short communication

Multiplex MassTag-PCR for respiratory pathogens in pediatric nasopharyngeal washes negative by conventional diagnostic testing shows a high prevalence of viruses belonging to a newly recognized rhinovirus clade

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ABSTRACT

Background: Respiratory infections are the most common infectious diseases in humans worldwide and are a leading cause of death in children less than 5 years of age.

Objectives: Identify candidate pathogens in pediatric patients with unexplained respiratory disease.

Study design: Forty-four nasopharyngeal washes collected during the 2004–2005 winter season from pediatric patients with respiratory illnesses that tested negative for 7 common respiratory pathogens by culture and direct immunofluorescence assays were analyzed by MassTag-PCR. To distinguish human enteroviruses (HEV) and rhinoviruses (HRV), samples positive for picornaviruses were further characterized by sequence analysis.

Results: Candidate pathogens were detected by MassTag PCR in 27 of the 44 (61%) specimens that previously were rated negative. Sixteen of these 27 specimens (59%) contained picornaviruses; of these 9 (57%) contained RNA of a recently discovered clade of rhinoviruses. Bocaviruses were detected in three patients by RT-PCR.

Conclusions: Our study confirms that multiplex MassTag-PCR enhances the detection of pathogens in clinical specimens, and shows that previously unrecognized rhinoviruses, that potentially form a species HRV-C, may cause a significant amount of pediatric respiratory disease.

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1. Introduction

Respiratory infections are the most common infectious diseases in humans worldwide and a leading cause of death in children less than 5 years of age.^{1–3} In the United States of America, acute viral respiratory infections (ARIs) are a significant cause of morbidity and hospitalization in children.^{4,5} Identification of previously unknown respiratory pathogens may lead to the development of new therapies and vaccines to treat or prevent ARIs.

Recently, a sensitive and highly multiplexed PCR platform, MassTag-PCR, was designed to address the limitations of conventional multiplex PCR.⁶ MassTag-PCR simultaneously detects 20–30

different viral and bacterial pathogens.^{6–8} Employing degenerate, genus-wide primers, MassTag-PCR also recently led to the discovery of a novel, rhinovirus-related clade of picornaviruses in association with influenza-like illness in New York State.⁷

We analyzed 44 specimens that tested negative for known respiratory pathogens by culture and direct immunofluorescence assays using MassTag-PCR and identified RNA from viral pathogens in 27 (61%) specimens, including RNA sequences in 9 (20%) specimens, that matched with a recently discovered novel clade of rhinoviruses. Our results show the importance of characterizing the role of these newly identified picornaviruses in pediatric respiratory disease.

2. Methods

As part of an ongoing epidemiological investigation of respiratory viruses at The Children's Hospital, Aurora, CO affiliated with

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the University of Colorado Denver School of Medicine (UCDSOM), we had archived at -70°C nasopharyngeal washes (NPWs) that tested negative for influenza viruses A and B (FLUAV and FLUBV), respiratory syncytial virus (RSV), parainfluenza viruses 1, 2 and 3 (HPIV-1, -2, -3), and adenovirus (HAdV) by virus culture and direct immunofluorescence assay (DFA). Forty-four such specimens (mean age \pm standard deviation (S.D.) 40.1 ± 33.4 months, 52% male, mean day of illness that specimens were collected 5.2 ± 4.5 days) obtained from pediatric patients with respiratory symptoms that had previously served as age- and season-matched controls in a study of coronavirus prevalence in Kawasaki disease⁹ were analyzed. Use of specimens and clinical data was approved by the Colorado Multiple Institutional Review Board.

Samples were prepared and analyzed by MassTag-PCR^{6,7} using primers that targeted FLUAV, FLUBV, RSV-A, RSV-B, human coronaviruses OC43 and 229E; HPIV 1-4; human metapneumovirus (HMPV); and human enteroviruses (HEV) and human rhinoviruses (HRV).⁶ Because a MassTag panel that includes human bocavirus (HBoV) primers is still in development, we also analyzed all samples by singleplex RT-PCR for HBoV.¹⁰

The degenerate HEV/HRV primer pair used in MassTag-PCR targets conserved motifs of the 5'-untranslated region present in both HEV and HRV.⁶ Samples positive for HEV/HRV by MassTag-PCR were therefore amplified for sequencing and genotyping by nested RT-PCR using degenerate primers that target the VP4/2 coding region.¹¹

Duplicate aliquots of all specimens transferred to Columbia University (CU) for analysis were kept at UCDSOM for conventional RT-PCR and sequencing, which independently confirmed MassTag-PCR results. Medical chart review was done to correlate clinical syndromes with the specimens analyzed.

3. Results

Forty-four NPWs collected between December, 2004, and July, 2005, were analyzed independently by MassTag-PCR at CU and by RT-PCR at CU and UCDSOM. MassTag-PCR identified at least one respiratory pathogen in 27 (61%) of the 44 specimens that had previously tested negative by culture and DFA (Table 1). Co-infections were found in 6 (14%) specimens. MassTag-PCR detected FLUBV

Table 1
Candidate pathogens found by MassTag-PCR and singleplex PCR in nasopharyngeal washes from pediatric patients with previously undiagnosed respiratory illnesses

	Number of patients (n = 44)	Percent
HRV-CO	9	20
HRV-A	5	11
HEV	2	5
HMPV	7	16
HBoV ^a	3	7
HPIV-2	1	2
HPIV-3	5	11
FLUBV	1	2
Coinfection	6	14
HRV-A + HPIV-3	1	2
HRV NY + HEV	1	2
HMPV + HPIV-2	1	2
HMPV + HBoV	1	2
HBoV + HPIV3	1	2
HPIV-3 + HEV	1	2
Patients positive for ≥ 1 pathogen	27	61
Patients negative	17	39

HRV: human rhinovirus, HRV-CO: human rhinovirus Colorado, HEV: human enterovirus, HMPV: human metapneumovirus, HBoV: human bocavirus, HPIV: human parainfluenza virus, FLUBV: influenza B virus.

^a Results based on conventional RT-PCR.

in 1, HPIV-2 in 1, and HPIV-3 in 5 patients, suggesting a greater sensitivity than the DFA and culture assays.

Conventional RT-PCR detected HBoV in 3 (7%) of 44 specimens. Two of these were co-infected with either HMPV or HPIV-3 (Table 1). The patient co-infected with HMPV was a 16-month-old girl with hypoxia and bronchiolitis hospitalized for 1 day. The patient co-infected with HPIV-3 was a 12-month-old boy seen in the emergency department on two occasions with fever but no other symptoms. HBoV was the sole identified agent in a 17-month-old boy admitted to the intensive care unit with respiratory failure.

HMPV was found in 7 (16%) specimens (Tables 1 and 2) from patients with a median age of 37 months who were all admitted during a six-week period from February 14 through March 30, 2005 with fever, cough, and hypoxia. Five (71%) had radiographic evidence of pneumonia.

Sixteen (36%) specimens were positive for picornaviruses, of which 14 were rhinoviruses. Sequencing of a 500 nucleotide region in their VP4/2 coding regions showed further that 5 of the 14 (36%) rhinovirus specimens contained HRV-A, while 9 (64%) of the sequences, HRV-Colorado (HRV-CO), matched with a recently described rhinovirus clade divergent from established HRV-A and -B.^{7,12–15} The demographic and clinical features of these patients are summarized in Table 2; amplified VP4/2 sequences are accessible under GenBank Acc. No. EU743920–EU743927. Four (44%) of the HRV-CO positive patients were under 2 years of age; and all but one patient (89%) were under 5 years of age. The positive specimens did not cluster in time: specimens were positive in the months of December, January, March, May, June and July, indicating prolonged circulation of these viruses in the community. Six (67%) of the 9 HRV-CO patients were hospitalized, and two required admission to the intensive care unit. One of these patients was a 3-year-old girl admitted for a severe asthma exacerbation and hypotension. The other patient was admitted for an accidental inhalation injury and subsequently developed fever, copious secretions, and respiratory distress necessitating ventilatory support. A chest X-ray taken at that time was consistent with pneumonia. The NPW obtained in hospital on day 6 was positive for HRV-CO, suggesting a recently acquired, possibly nosocomial infection.

Although 7 (78%) of the 9 patients with HRV-CO had typical upper respiratory tract infection symptoms including rhinorrhea, cough, and congestion, 5 (56%) also had respiratory distress (retractions), 3 (33%) were hypoxic, and 4 (44%) were wheezing. One patient undergoing chemotherapy for anaplastic lymphoma was admitted with a diagnosis of fever and neutropenia. Another patient with severe cerebral palsy was admitted with clinical signs of respiratory distress and hypoxia.

4. Discussion

Rapid identification of the causative agent of an infectious disease can affect clinical management and have important public health implications. Accurate data concerning the burden of disease caused by specific agents are essential for prioritizing targets for therapeutic interventions and vaccine development. Here, we report the use of MassTag-PCR to investigate ARI during the winter 2004–2005 season in pediatric patients from Denver, Colorado, that remained without diagnosis after DFA and culture. In 61% of these 44 samples a viral pathogen was identified by MassTag-PCR.

HRVs are the most commonly implicated viruses in upper respiratory tract infections in both adults and children,^{16,17} but may also cause bronchiolitis, asthma exacerbations, and pneumonia.^{15,18–23} In a recent population-based study of hospitalized children under 5 years of age, the incidence of HRV infection was 5/1000. Interestingly, 26% of children hospitalized with respiratory symptoms or fever tested positive for HRV; pneumonia was diagnosed in 16%

Table 2

Clinical summaries of pediatric patients with respiratory disease in whom human metapneumovirus (HMPV), human rhinovirus A (HRV-A), and a recently discovered rhinovirus clade (HRV-CO) were detected by MassTag-PCR

	HMPV (n = 7) Number (%)	HRV-A (n = 5) Number (%)	HRV-CO (n = 9) Number (%)
Children			
Specimen dates	2/14/05–3/30/05	12/18/04–6/25/05	12/20/04–7/1/05
Male	3 (43)	2 (40)	6 (67)
Median age in months (range)	37 (16–59)	21 (1.5–28)	25 (4–84)
Hospitalized	7 (100)	4 (80)	6 (67)
ICU admission	1 (14)	1 (20)	2 (22)
Duration of hospitalization—median (range)	3 (1–18)	5 (0–6)	2 (2–30)
Underlying illness	4 (57)	2 (40)	4 (44)
Sick contacts	1 (14)	1 (20)	1 (11)
Clinical symptoms			
Fever	7 (100)	4 (80)	4 (44)
Rhinorrhea	3 (43)	4 (60)	7 (78)
Cough	7 (100)	3 (60)	7 (78)
Hypoxia	7 (100)	3 (60)	3 (33)
Retractions	5 (71)	3 (60)	5 (56)
Wheezing	1 (14)	2 (40)	4 (44)
Abnormal chest X-ray (+infiltrates)	5 (72)	2 (40)	3 (33)
Clinical diagnosis			
Otitis	0	1 (20)	0
Pharyngitis	0	0	2 (22)
Bronchiolitis	2 (29)	2 (40)	1 (11)
Pneumonia	5 (71)	1 (20)	2 (22)
Asthma exacerbation	2 (29)	0	2 (22)
Viral syndrome	0	1 (20)	0
Seizures	0	2 (40)	0
Other	0	0	4 (44)
Coinfection	2 (29)	1 (20)	1 (11)
Treatment			
Antibiotics	6 (86)	4 (80)	5 (56)
Albuterol	6 (86)	2 (40)	4 (44)
Steroids	3 (43)	0	3 (33)

of HRV patients, whereas 25% of febrile neonates positive for HRV did not show any clinical signs of respiratory disease.²⁰ A recent prospective study of acute upper and lower respiratory tract illnesses in the first year of life found that HRVs were associated with 10 times more upper respiratory tract disease and 3 times more wheezing and lower respiratory tract disease than RSV.²⁴

Nine of the 14 (64%) HRVs detected in our study belonged to a novel genotype recently discovered in respiratory specimens from patients with influenza-like illnesses in New York State.⁷ These viruses appear to be closely related to viruses recently reported in association with respiratory illnesses from Australia,¹² USA,¹⁵ and China.¹³

Our findings confirm the power of multiplex MassTag-PCR as a diagnostic tool and show that a recently discovered clade of picornaviruses,^{7,12–15} that may ultimately be classified by the International Committee on Taxonomy of Viruses as a third species ('HRV-C') of rhinoviruses, is associated with significant respiratory disease in pediatric patients, indicating the need for further epidemiological studies of this virus.

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